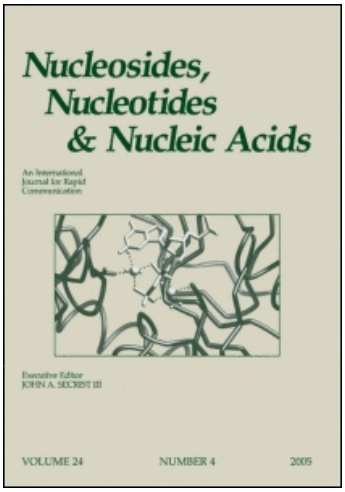


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# Synthesis of Cholesteryl Supports and Phosphoramidite for Automated DNA Synthesis of Triple-Helix Forming Oligonucleotides (Tfos)

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# SYNTHESIS OF CHOLESTERYL SUPPORTS AND PHOSPHORAMIDITE FOR AUTOMATED DNA SYNTHESIS OF TRIPLE-HELIX FORMING OLIGONUCLEOTIDES (TFOs).

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**ABSTRACT:** DMT-Cholesteryl succinylamino solid supports (CPG, loading: 33 μmole/gram and TentaGel loading: 152 μmole/gram) and DMT-cholesteryl phosphoramidite were prepared for use in automated DNA synthesis of cholesteryl modified TFOs in the synthesis scales from 0.2 to 300 μmole. The modified TFOs were found to have a 5 to 50 fold increase in their uptake properties.

Oligonucleotides have been shown to be taken up by mammalian cells rather easily[1]. Uptake has been shown to result in nuclear accumulation[2]. Attachment of lipophilic molecules to oligonucleotides was shown to increase their uptake properties considerably[3-9]. Specifically, attachment of cholesterol and poly-L-lysine have been studied in detail[4,9]. Our goal is to enhance the cellular uptake and therapeutic efficacy of sequence specific Triple-Helix Forming Oligonucleotides (TFOs) by attaching lipophilic groups, such as cholesterol. Attachment of cholesterol to TFOs has been accomplished using either cholesteryl supports or cholesteryl phosphoramidite.

The synthesis of cholesteryl supports (6, 7) and cholesteryl phosphoramidite (8) using 3-aminopropylsolketal (2) is described in FIG. I. (2) was prepared according to Gait et al[10]. Cholesteryl chloroformate (1) was reacted with compound 2 at room temperature under basic condition to yield N-(cholesteryloxycarbonyl)-3-aminopropylsolketal (3). The ketal protecting group on compound 3 was removed by treatment with 1N HCl solution. The product

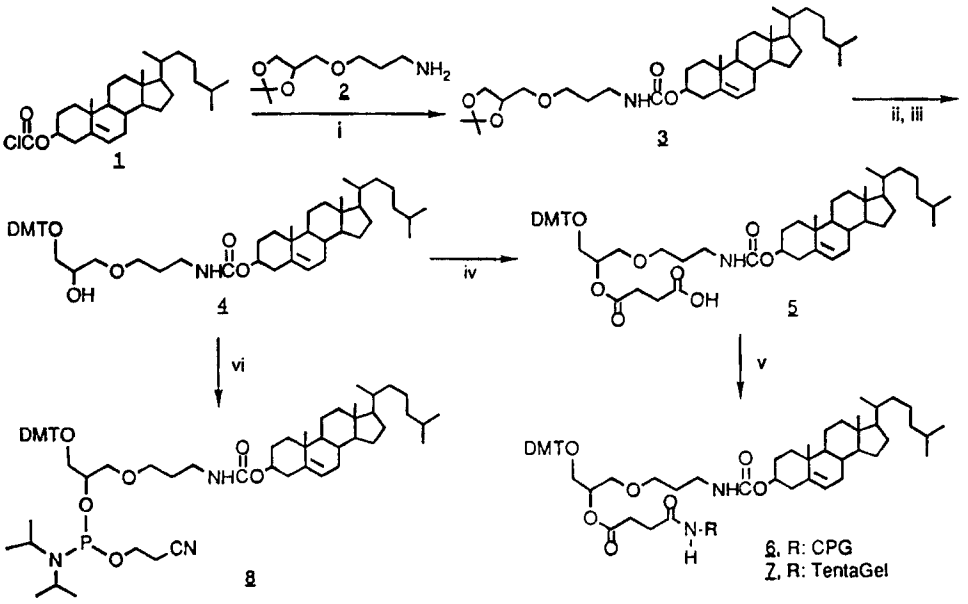


FIG. I: i. pyridine; ii. HCl, MeOH, $CH_2Cl_2$; iii. 4',4'-dimethoxytrityl chloride, pyridine; iv. succinic anhydride, DMAP, pyridine; v. TBTU, HOBT, DMF, N-ethylmorpholine; vi. 2-cyanoethyl N,N-diisopropyl chloro-phosphoramidite, DIPEA, $CH_2Cl_2$.



was then tritylated selectively on primary hydroxyl group to give 1-O-(4,4'-dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl)glycerol (4) by 4,4'-dimethoxytrityl chloride in the presence of pyridine. Compound 4 was succinylated by succinic anhydride and DMAP to provide 1-O-(4,4'-dimethoxytrityl)-2-O-succinate-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl) glycerol (5) which was coupled to free amino groups on two different kind of solid supports CPG (6, loading : 33 μmole/gram) and TentaGel (7, loading: 152 μmole/gram) by using O-(1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU). Cholesteryl derivative (4) was converted to 1-O-(4,4'-dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl)glyceryl 2-cyanoethyl-N,N-diisopropyl phosphoramidite (8) by treatment with 2-cyanoethyl-N,N-diisopropylaminochlorophosphine. $^{31}P$ NMR ($CDCl_3$) : $\partial$ 149.82 and 149.69 ppm (FIG. II).

## EXPERIMENTAL

**Synthesis of N-(Cholesteryloxycarbonyl)-3-aminopropylsolketal (3).** 3-Amino-propylsolketal (2, 5.0 g, 26.4 mmole) was dissolved in anhydrous pyridine (50

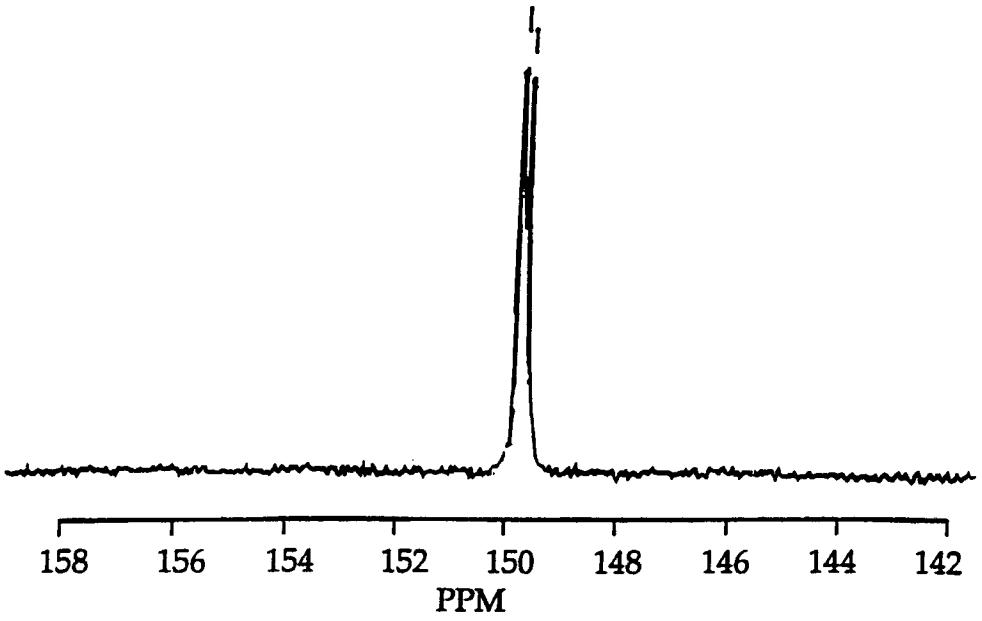


FIG. II: $^{31}$P-NMR of cholesteryl phosphoramidite 7



mL, Aldrich Chem. Co.), and the solution was cooled in an ice cold water bath. To the stirred solution was added cholesteryl chloroformate (1, 11.87 g, 26.4 mmole, Aldrich Chem. Co.). The reaction mixture was continued stirring for 15 minutes at cold temperature and 3 hours at room temperature. The resulting reaction mixture was concentrated to a heavy oil to remove pyridine. The oil was re-dissolved in dichloromethane (250 mL), washed with saturated aqueous sodium bicarbonate solution (200 mL, twice) and, brine (200 mL, twice). The organic phase was dried over anhydrous $Na_2SO_4$. After removal of solvent, the crude oil was purified by silica gel column chromatography using a gradient of $CH_2Cl_2$ and $CH_2Cl_2$ : MeOH (19 : 1), as the eluant. The homogeneous fractions were combined and concentrated under reduced pressure to provide product 3 (13.98 g, yield : 85%) as a light yellow solid foam. $^1$H NMR ($CDCl_3$): ∂ 0.68 (s, 3H, -C<u>H</u>$_3$, chol.), 0.86 (d, J=6.8Hz, 3H, -C<u>H</u>$_3$, chol.), 0.87 (d, J=6.8Hz, 3H, -C<u>H</u>$_3$, chol.), 0.91 (d, J=6.68Hz, 3H, -C<u>H</u>$_3$, chol.), 0.99 (s, 3H,-C<u>H</u>$_3$, chol.), 1.36 and 1.43(s,s, 6H, C(C<u>H</u>$_3$)$_2$), 2.59-0.95 (m, 29H, cholesterol), 1.73 (quintet, J=6.08Hz, 2H, C-C<u>H</u>$_2$-C, linker), 3.23-3.16 (m, 4H, 2(C<u>H</u>$_2$), linker), 3.53-3.46 (m, 4H, 2(C<u>H</u>$_2$),

linker), 3.95 (m, H, $C_2$=C<u>H</u>-O-, linker), 4.47 (m, H, chol.), 5.35 (d, J=4.72, H, C<u>H</u>=C-, chol.).

**Synthesis of 1-O-(4,4'-dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl)glycerol (4).** N-(Cholesteryloxycarbonyl)-3-aminopropylsolketal (3, 3.1 g, 5.15 mmole) was dissolved in 1N HCl (10 mL), methanol (10 mL) and dichloromethane (10 mL). The mixture was stirred at room temperature for 1 hour. The reaction mixture was concentrated to dryness and co-evaporated with anhydrous pyridine (25 mL, 3 times) to provide a light yellow oil which was re-dissolved in pyridine (30 mL). To the solution was added 4,4'-dimethoxytrityl chloride (2.09 g, 6.18 mmole, Aldrich Chem. Co.). The solution was stirred at room temperature for 1 hour and then methanol (10 mL) was added. The resulting mixture was concentrated to dryness to remove pyridine and methanol and was re-dissolved in dichloromethane (200 mL). It was then washed with saturated $NaHCO_3$ aqueous solution (100 mL, twice) and brine (100 mL, twice ). The organic phase was dried over anhydrous $Na_2SO_4$ and concentrated to give a light yellow oil (4.7 g). The crude oil was purified by silica gel column chromatography using a gradient of $CH_2Cl_2$ : EtOAc (1 : 1) and $CH_2Cl_2$ , as the eluant. The homogeneous fractions were combined and concentrated under reduced pressure to provide product 4 (2.96 g, yield : 66.4%) as a light yellow solid foam. $^1$H NMR ($CDCl_3$): $\partial$ 0.68 (s, 3H, -C<u>H</u>3, chol.), 0.86 (s, 3H, -C<u>H</u>3, chol.), 0.87 (d, J=1.9Hz, 3H, -C<u>H</u>3, chol.), 0.91 (d, J=6.68Hz, 3H, -C<u>H</u>3, chol.), 0.99 (s, 3H,-C<u>H</u>3, chol.), 2.59-0.95 (m, 29H, cholesterol), 1.73 (quintet, J=6.08Hz, 2H, C-C<u>H</u>2-C, linker), 3.23-3.16 (m, 4H, 2(C<u>H</u>2), linker), 3.53-3.46 (m, 4H, 2(-C<u>H</u>2), linker), 3.78 (s, 6H, 2(O-C<u>H</u>3), DMT), 3.95 (m, H, $C_2$=C<u>H</u>-O-, linker), 4.47 (m, H, chol.), 5.35 (d, J=4.72, H, C<u>H</u>=C-, chol.), 7.42-6.80 (m, 13H, DMT).

**Synthesis of 1-O-(4,4'-dimethoxytrityl)-2-O-succinate-3-O-(N-(cholesteryloxy carbonyl)-3-aminopropyl)glycerol (5).** 1-O-(4,4'-Dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl)glycerol (4, 2.96 g, 3.42 mmole) was dissolved in anhydrous pyridine (30 mL). To the solution was added succinic anhydride (0.51 g, 5.13 mmole, Fluka Chem. Co.), and 4-dimethylaminopyridine (0.33 g, 2.7 mmole, Aldrich Chem. Co.). The mixture was stirred for 2 hours and then was concentrated to an oil to remove pyridine. The oil was re-dissolved in dichloromethane (200 mL), washed with 10% aqueous citric acid solution (150 mL, twice) and water (150 mL, twice). The organic phase was dried over anhydrous $Na_2SO_4$. After concentration, the crude oil was purified by silica gel

column chromatography using a gradient of $CH_2Cl_2$ and $CH_2Cl_2$ : MeOH (19 : 1), as the eluant. The homogeneous fractions were combined and concentrated under reduced pressure to provide the product 5 (2.34 g, yield : 70%) as a white solid foam. $^1H$ NMR ($CDCl_3$): $\partial$ 0.68 (s, 3H, -C<u>H</u>$_3$, chol.), 0.86 (d, J=6.8Hz, 3H, -C<u>H</u>$_3$, chol.), 0.87 (d, J=6.8Hz, 3H, -C<u>H</u>$_3$, chol.), 0.91 (d, J=6.68Hz, 3H, -C<u>H</u>$_3$, chol.), 0.99 (s, 3H,-C<u>H</u>$_3$, chol.), 2.59-0.95 (m, 29H, cholesterol), 1.73 (quintet, J=6.08Hz, 2H, C-C<u>H</u>$_2$-C, linker), 2.68 (br s, 4H, 2($CH_2$), succinyl), 3.23-3.16 (m, 4H, 2(C<u>H</u>$_2$), linker), 3.53-3.46 (m, 4H, 2(-C<u>H</u>$_2$), linker), 3.78 (s, 6H, 2(O-C<u>H</u>$_3$), DMT), 3.95 (m, H, $C_2$=C<u>H</u>-O-, linker), 4.47 (m, H, chol.), 5.35 (d, J=4.72, H, C<u>H</u>=C-, chol.), 7.42-6.80 (m, 13H, DMT).

**Synthesis of Cholesterol-CPG Support (6).**

**Procedure 1:** 1-O-(4,4'-Dimethoxytrityl)-2-O-succinate-3-O-(N-(cholesteryloxy carbonyl)-3-aminopropyl)glycerol (5, 0.49 g , 0.5 mmole) was dissolved in anhydrous dioxane (8 mL, Aldrich Chem. Co.) containing 0.4 mL of pyridine. To the solution was added p-nitrophenol (60 mg, 0.5 mmole, Aldrich Chem. Co.), and 1,3-dicyclohexylcarbodiimide (200 mg, 1 mmole, Aldrich Chem. Co.). After stirring for 1 hour, the reaction mixture was filtered to separate N,N'-dicyclohexylurea and the filtrate was added to aminopropyl CPG (1 g, loading capacity : 37 μmole/g) in anhydrous DMF (10 mL, Aldrich Chem. Co.). Triethylamine (2 mL) was added to the reaction mixture which was swirled slowly overnight, at room temperature. Capping reagents A and B (15 mL each, MilliGen Inc.) were then added to the reaction mixture. The mixture was continued swirling for 30 minutes to cap the unreacted free amino groups on support. Cholesterol-CPG support was collected by filtration, washed with DMF (5 mL, 3 times), methanol (5 mL, 3 times) and ether (5 mL, 3 times) and dried on a high vacuum pump for overnight. Cholesterol loading was estimated by the measurement of trityl cation released by acidic treatment of synthesized support, and was estimated to be 18 μmole/g.

**Procedure 2 :** 1-O-(4,4'-Dimethoxytrityl)-2-O-succinate-3-O-(N-(cholesteryloxy-carbonyl)-3-aminopropyl)glycerol (5, 1.62 g, 1.65 mmole), O-benzotriazole-1-yl-N,N,N',N'-tetramethyluronium tetrafluoroborate (0.53 g, 1.65 mmole, Fluka Chem. Co.), 1-hydroxy benzotriazole hydrate (0.22 g, 1.65 mmole, Fluka Chem. Co.), and 4-ethylmorpholine (0.19 g, 1.65 mmole, Fluka Chem. Co.) were dissolved in anhydrous DMF (25 mL). After 5 minutes of shaking, to the mixture was added aminopropyl CPG (3 g, loading capacity : 37 μmole/g). The reaction

mixture was swirled slowly for 3 hours. Capping and washing steps were done as described above. The loading of cholesterol was estimated to be 33 μmole/g.

**Synthesis of Cholesterol-TentaGel Support (7).** 1-O-(4,4'-Dimethoxytrityl)-2-O-succinate-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl)glycerol (5, 3.2 g, 3.26 mmole), O-benzotriazole-1-yl-N,N,N',N'-tetramethyluroniumtetrafluoroborate (1.05 g, 3.26 mmole, Fluka Chem. Co.), 1-hydroxy benzotriazole hydrate (0.44 g, 3.26 mmole , Fluka Chem. Co.), 4-ethylmorpholine (0.37 g, 3.26 mmole , Fluka Chem. Co.) were dissolved in anhydrous DMF (76 mL). After 5 minutes of shaking, TentaGel-$NH_2$ (10 g, loading capacity : 220 μmole/g) was added to the mixture. The reaction mixture was swirled slowly for 3 hours. Capping reagents A and B (15 mL each, MilliGen Inc.) were then added to the reaction mixture. The mixture was continued swirling for 30 minutes to cap the unreacted free amino groups on support. Cholesterol-TentaGel support was collected by filtration, washed with DMF (5 mL, 3 times), methanol (5 mL, 3 times) and ether (5 mL, 3 times) and dried on a high vacuum pump overnight. Cholesterol loading was estimated to be 152 μmole/gram.



**Synthesis of 1-O-(4,4'-dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl)glyceryl 2-cyanoethyl N,N-diisopropylphosphoramidite (8).** 1-O-(4,4'-Dimethoxytrityl)-3-O-(N-(cholesteryloxycarbonyl)-3-aminopropyl) glycerol (4 ,1.5 g, 1.74 mmole) was dissolved in distilled dichloromethane (15 mL). To the solution was added distilled N,N'-diisopropylethylamine (0.45 g, 3.48 mmole) and 2-cyanoethyl-N,N'-diisopropylaminochlorophosphine (0.45 g, 1.9 mmole, Aldrich Chem. Co.). The mixture was stirred for 1 hour at room temperature, under argon atmosphere. After stirring, the reaction mixture was diluted with 50 mL of $CH_2Cl_2$ and washed with 5% $NaHCO_3$ aqueous solution (50 mL, twice), and brine (50 mL, twice ). The organic phase was dried over anhydrous $MgSO_4$ and concentrated to give a light yellow oil (2.3 g). The crude oil was purified on a silica gel column using ethyl acetate: dichloromethane: triethylamine (49: 49: 2) solution as the eluant. The homogeneous fractions were combined and concentrated under reduced pressure to provide the product, 8 (1.3 g, yield : 70%) as a white solid foam. $^{31}P$ NMR ($CDCl_3$) : ∂ 149.82 and 149.69; $^{1}H$ NMR ($CDCl_3$): ∂ 0.67 (s, 3H, -C<u>H</u>$_3$, chol.), 0.85 (s, 3H, -C<u>H</u>$_3$, chol.), 0.87 (s, 3H, -C<u>H</u>$_3$, chol.), 0.91 (d, J=6.68Hz, 3H, -C<u>H</u>$_3$, chol.), 1.0 (s, 3H,-C<u>H</u>$_3$, chol.), 1.14-1.20 (m, 12H, diisopropyl), 2.59-0.95 (m, 29H, cholesterol), 1.73 (quintet, J=6.08Hz, 2H, C-C<u>H</u>$_2$-C, linker), 2.6(m, 2H, $CH_2$-O-P), 3.23-3.66 (m, 6H, 2(C<u>H</u>$_2$), linker, $CH_2CN$),

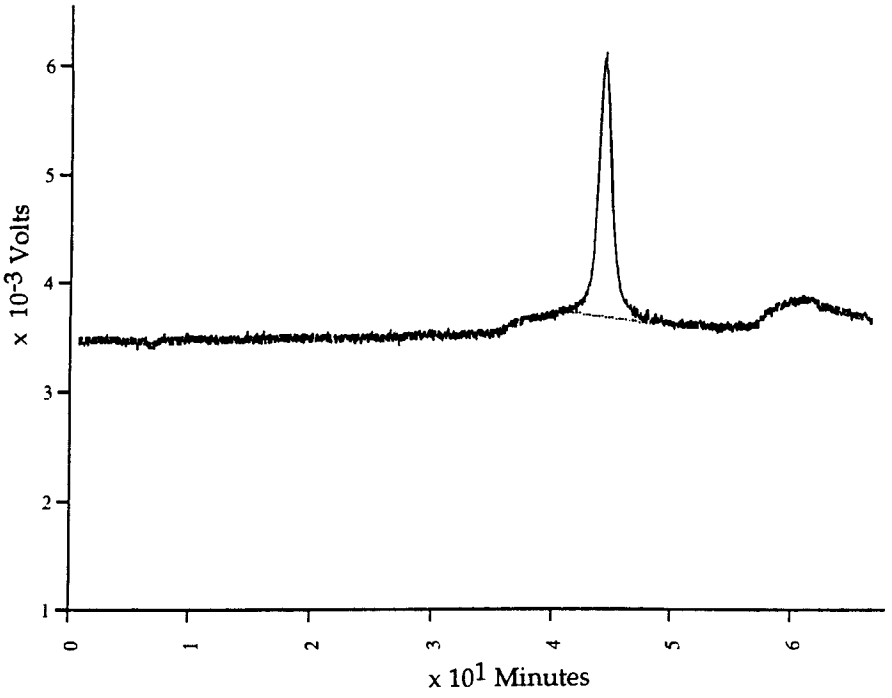


FIG. III: Analytical ion-exchange HPLC of purified oligo 2 (38mer, with 3'cholesterol). Buffer A: 0.5M NaCl; 10mM NaOH, buffer B: 1.5 M NaCl; 10mM NaOH. Column: high performance Q Sepharose, flow rate: 2.5 ml/min., gradient: 0-55 min., 80-30% A and 20-70% B.



3.53-3.46 (m, 4H, 2(-C<u>H</u>$_2$), linker), 3.78 (s, 6H, 2(O-C<u>H</u>$_3$), DMT), 3.95 (m, H, C$_2$=C<u>H</u>-O-, linker), 4.47 (m, H, chol.), 5.35 (d, J=4.72, H, C<u>H</u>=C-, chol.), 7.42-6.80 (m, 13H, DMT).

**Oligonucleotide synthesis and purification:** Several 3' end cholesteryl modified TFO sequences were synthesized from 0.2 to 300 μmole scale using supports <u>6</u> and <u>7</u> on Applied Biosystems Models 380B , 392/4 and or MilliGen Models 8700 and 8800 with a coupling efficiency of ≥ 97%. Two representative examples are shown below:

5' GTGTGTTGTGGGGGGTGGGGGTGTTGTGTGTGTTGTC 3' cholesterol --- oligo 1

5' TGGGTGGGGTGGGGTGGGGGGGTGTGGGGTGTGGGGTG 3' cholesterol --- oligo 2

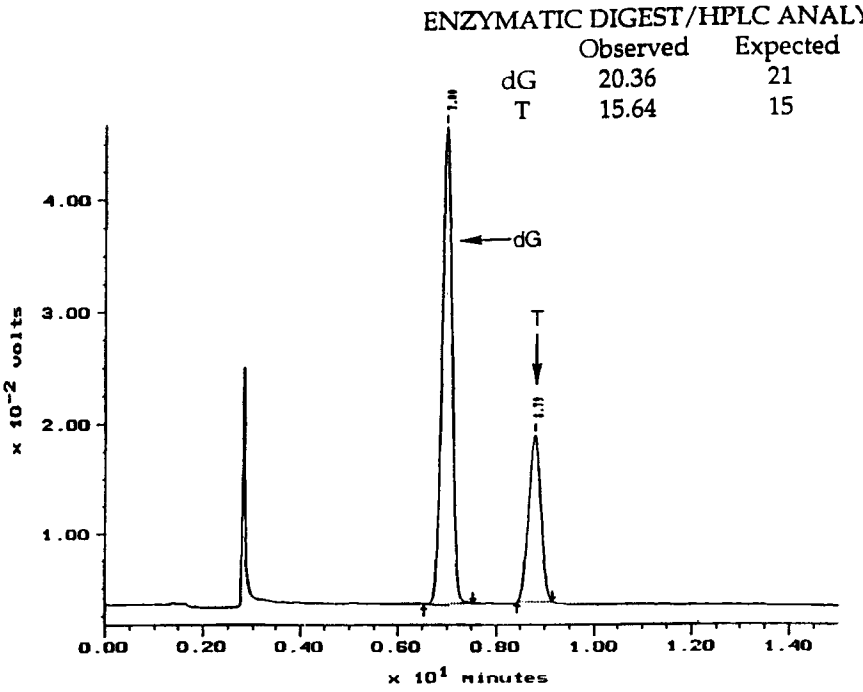


FIG. IV: Base composition analysis of oligo 2 degraded by P1 nuclease/bacterial alkaline phosphatase treatment. The resulting deoxynucleosides were separated on a reverse phase column (Customsil ODS, 4.6x250mm). Buffer A: water, buffer B: acetonitrile, flow rate: 1.0 ml/min., gradient: 0-35 min., using an isocratic system with 98% A and 2% B.

Cleavage and deprotection were carried out under standard conditions. Crude oligonucleotides were purified on a Pharmacia FPLC system by anion exchange chromatography on a Q-Sepharose column (1 cm x 10 cm). The elution time of cholesteryl modified TFOs was about 15 minutes higher than unmodified TFOs as shown in FIG. III. Enzymatic digestion of the cholesteryl oligonucleotides by P1 nuclease/bacterial alkaline phosphatase gave the expected deoxynucleoside composition. The linker containing cholesterol is attached to the oligonucleotide through a phosphate linkage and so the end 3' base is likely to be cleaved from the linker-cholesterol during P1 nuclease digestion. As a result, base composition analysis will yield only dGs and Ts. The presence of cholesterol in these compounds can not be verified by this procedure. The results are shown in FIG. IV.

Gel electrophoresis analysis of 5'-end $^{32}P$ labeled cholesteryl oligonucleotides using polynucleotide kinase showed that oligonucleotide containing cholesterol at the 3' end migrated one unit slower than the oligos containing 3' end free amino group (oligonucleotides containing 3' propanol amine group were synthesized using 3' amine CPG obtained from Glen Research Corporation). In the case of oligo-dT (19 mer) the difference in mobility was much greater (data not shown). 5' end cholesteryl modified TFOs were also synthesized in small scale syntheses using cholesteryl phosphoramidite 8. However, the coupling yield of 8 to the 5' hydroxyl of the support bound oligonucleotides was low (~ 50%), even when the coupling time was extended to 300 seconds[12]. Interestingly, in the synthesis of the sequence containing two cholesterols at the 3' end, the coupling of 8 on the support bound cholesterol gave a stepwise trityl yield of 97.5%.



## CELLULAR UPTAKE

**a) Radiolabeling of Oligonucleotides.** Prior to uptake experiments oligonucleotides were radiolabeled at the 5' end using polynucleotide kinase and $\gamma^{35}S$-ATP (Amersham). Oligonucleotide was separated from unreacted ATP by chromatography on a Sephadex G-100 column (Pharmacia) 1 x 30 cm.

**b) Cell Culture.** SKBR3 and MDA-MB-361 cell lines were a gift of Dr. Stuart Aaronson at NCI and were maintained in Modified Eagles Medium with 10% heat inactivated fetal bovine serum (FBS) at 37°C in an atmosphere of 95% air/5% $CO_2$.

The $^{35}S$-label was found to be intact and was associated with the TFOs at least up to 48 hours when the labeled oligonucleotides were incubated with FCS[13] or alkaline phosphatase (data not shown).

For cell uptake experiments, cells were grown to near confluence in 75cm$^2$ Corning tissue culture flasks, trypsinized and transferred to 24 well dishes at a density of 100,000 cells per well. After incubation for 24 hours at 37°C $2x10^6$ cpm of labeled oligonucleotide was added to each well and unlabeled oligonucleotide was added to a final concentration of 1μM. Cells were harvested at several points between 1 hour and 24 hours as follows. Wells were rinsed 6x with MEM, suctioning between rinses. The cells were detached from the wells by careful trypsinization and immediately resuspended in 600 μl MEM + 10% FBS. The resuspended cells were transferred to 1.5 mL tube and spun in a microcentrifuge at 1000xg at room temperature for 6 minutes to pellet. The supernatant was removed and discarded and the pellet was resuspended in

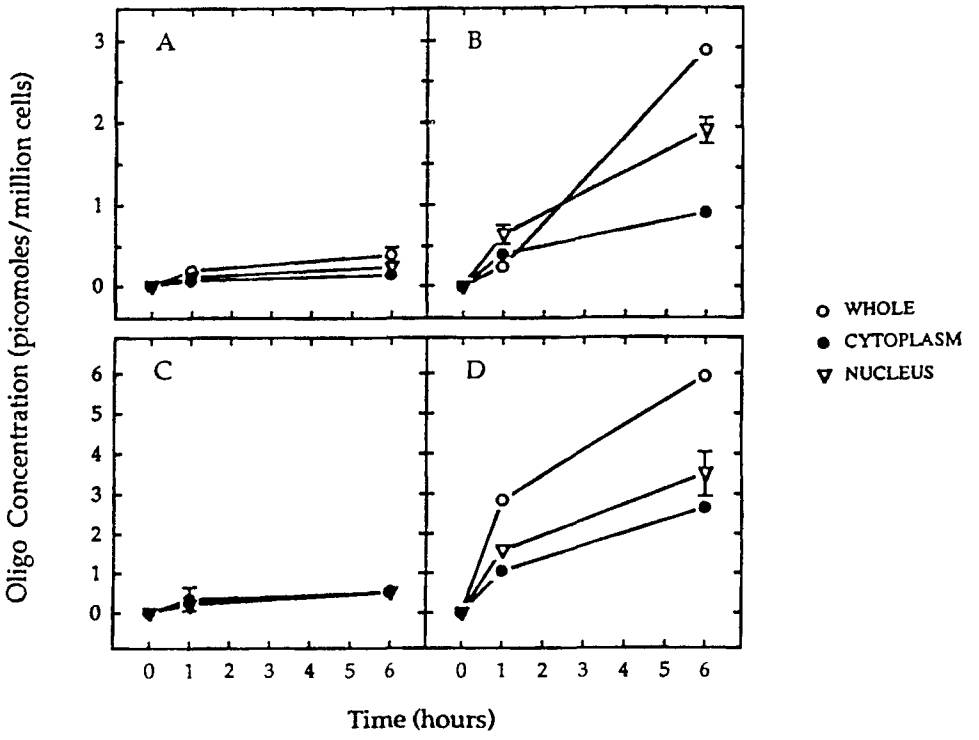


FIG. V. Cellular and nuclear uptake of oligo 2 (3' amine, instead of 3'cholesterol, Panels A & C) and oligo 2 (3'cholesterol, Panel B & D) in MDA-MB-361 cells (Panels A & B) and SKBR3 cells (Panels C & D). Each panel shows the accumulation of labeled oligonucleotide in whole cells (o), and in nuclear (▽), and cytoplasmic (•) fractions.



500 µl of MEM and recetrifuged. For "whole cell" determinations the pellet was resuspended in 500 µl PBS and removed to a scintillation counting vial. For cell fractions the pellet was resuspended in 500 µl lysing buffer at 4° and incubated on ice for 5 minutes centrifuged as above and the pellet (nuclei) and supernatant (cytoplasm) were separated. The nuclear pellet fraction was washed again with lysing buffer and resuspended in 500 µl. All samples are then analyzed by liquid scintillation counting.

Previous studies have shown that the labeled materials recovered from cytoplasmic, nuclear and whole cell fractions were associated with intact oligonucleotides at least up to 24 hours[14,15].

Cellular uptake studies were carried out with several TFOs with and without cholesterol at the 3' end. These results demonstrated that 3'-amine modified oligonucleotides are accumulated by all cells such that the internal concentration is equal to or exceeds that of the culture media (1 μM). Cholesterol modified oligonucleotides, however, reach concentrations that exceed that of the media by 5-50 fold. FIG. V shows the uptake of oligo 2 (3' amine or cholesterol) in MDA-MB-361 and SKBR3 cells.

The results show approximately a 5 to 10 fold increase in the uptake as a result of attaching cholesterol to the TFO. The level of enhancement seems to vary with the cell line. The 3' amine modified TFOs were quite stable for at least 8 hours *in vitro* and *in vivo*[16] and there should be no difference in stability between cholesteryl and amine modified TFOs at least during the period studied for uptake.



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